REMARKS

Favorable reconsideration of the subject application as amended above is respectfully requested in view of the following comments.

Claims 1-7 and 9-16 are pending in the present application; claim 8 having been canceled; and claims 11-14 withdrawn from consideration. Accordingly, claims 1-7, 9, 10, 15 and 16 are presented for examination on the merits.

Claim 1 has been amended to recite that the buffered medium comprises trypsin, a trypsin substrate and polycarboxylic chelating agent. The claim has been further amended to recite that the chelating agent reduces variation in the detected amount of trypsin inhibitor compared to control lacking the chelating agent. Support for this latter amendment is found throughout the specification, and in particular Tables A and B and the summary of results shown in Tables A and B set forth on page 9. Accordingly, no new matter is added by these amendments to claim 1.

It is respectfully submitted that the amendments to claim 1 do not add new matter and do not necessitate additional searching on the part of the Examiner. Moreover, these amendments place the claims in condition for allowance. As such, it is respectfully submitted that the amendments are proper and should be entered.

I. Rejection of Claims 1-7, 9, 10, 15 and 16 Under 35 U.S.C. § 112

Claims 1-7, 9, 10, 15 and 16 stand rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the written description requirement. It is respectfully submitted that this rejection is rendered moot by the amendments to claim 1 above.
Accordingly, it is respectfully requested that this rejection be withdrawn.

II. Rejection of Claims 1-7, 9, 10, 15 and 16 Under 35 U.S.C. § 112

Claims 1-7, 9, 10, 15 and 16 are rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the written description requirement. It is respectfully submitted that this rejection is rendered moot by the amendments to claim 1 above.

Accordingly, it is respectfully requested that this rejection be withdrawn.

III. Rejection of Claims 1-7, 9, 10, 15 and 16 Under 35 U.S.C. § 112

Claims 1-7, 9, 10, 15 and 16 are rejected under 35 U.S.C. § 112, second paragraph as allegedly being indefinite. It is respectfully submitted that the amendments to claim 1 render this rejection moot. Withdrawal of this formal ground of rejection is respectfully requested.

IV. Rejection of Claims 1-4, 7, 9, 15 and 16 Under 35 U.S.C. § 103(a)

Claims 14, 7, 9, 15 and 16 stand rejected under 35 U.S.C. § 103(a) as being unpatentably obvious over Uenoyama et al. in view of Berry et al. The Examiner states that the primary reference merely differs from the present invention in that it fails to teach the addition of a polycarboxylic chelating agent to the buffered medium to inhibit interference with calcium present in the medium. The Examiner relies on the secondary reference (Berry et al.) as teaching the use of EGTA or EDTA as chelating agents which inhibit interfering calcium ions in a urine sample. The Examiner concludes, therefore, that it would have been obvious to one of ordinary skill in the art to incorporate the polycarboxylic chelating agent of the secondary reference into the method taught by Uenoyama.

Applicant respectfully disagrees with the Examiner's conclusion.

The present invention is directed to a method for eliminating the interference of calcium ions with trypsin activity so that trypsin inhibitor activity in a urine sample can be accurately measured. Applicants have discovered that certain chelators, the polycarboxylic chelators, such as EDTA and EGTA, bind sufficiently strongly to calcium ions to completely cage the ion and prevent it from binding to and interfering with trypsin, without the necessity of having to remove the chelator-calcium complex from the reaction buffer. Applicants' studies have shown that this effect of polycarboxylic chelators is specific to trypsin. This effect is not seen with other enzymes. Moreover, Applicants' studies have shown that other chelators, such as monochelator phosphates, are capable of binding to calcium ions, but are not capable of inhibiting calcium from binding to trypsin and interfering with its activity.

Applicant tested buffer systems containing 50mM sodium dihydrogen phosphate, a known monochelator phosphate to determine whether this chelator has an inhibitory effect on calcium ions. The results are reported in Table A on page 8 (Example 1), where it is seen that there is significant variation between samples in the presence of this monochelator. However, when approximately 0.002 M EGTA was added to the buffer system, variation between samples was significantly reduced.

The results reported in Tables A and B also demonstrate that addition of EGTA to a urine sample containing trypsin does not inhibit trypsin activity. This result is surprising since it is known that most enzymes are sensitive to selective binding agents, such as chelators. For example, it is well known in the art that the enzyme, alkaline phosphatase, is inhibited by EGTA (Zygowitz, E. 1975, copy enclosed; Shan et al., 1983, Anal. Chem., 65:3053-3060, copy enclosed). Thus, Applicants' studies demonstrate for the first time that addition of polycarboxylic chelators to a sample can prevent calcium from interfering with trypsin activity without causing any reduction in

trypsin activity. This discovery has enabled Applicants to develop an accurate and easy to use test for the presence of trypsin inhibitor in a urine sample.

In contrast to the present invention, the primary reference merely discloses a method for assaying amount of trypsin inhibitor in a urine sample which requires **the addition of calcium** to the assay medium. According to Uenoyama et al., "when the concentration of the calcium mixed in the buffer solution is or the like is low, trypsin may be activated by the influence of calcium in the urine sample, so that the observed trypsin activity measurement would indicate a lower value for the urinary trypsin inhibitor concentration than the real value. Furthermore, if an excess amount of calcium is added, it reacts with carbonate ions, phosphate ions and the like present in the urine to produce precipitates, which affect the measurement." (Col. 1:41-49) Uenoyama et al. teaches that by holding the calcium concentration at a constant level in the assay buffer, a reproducible value for trypsin inhibitor is obtained. Uenoyama et al. teach that **calcium must be added to the assay buffer** in a range of from 0.15 micomol or more per 1 microgram of trypsin in order for the activity of the trypsin to be constant. For example, Uenoyama et al. disclose:

> A further aspect of the invention is the use of a particular calcium content in the assay mixture. Thus, in this aspect, the invention provides a method for the assay of a protease inhibitor in a sample, comprising mixing the sample, a protease, calcium and a protease substrate, and assaying the content of protease inhibitor in the sample by measuring the activity of the protease, characterized in that, the calcium content is at least 0.15 .mu.mol per 1 .mu.g of protease and no more than 100 .mu.mol per 1 ml of the sample. (Col. 2:38-46)

and

> The calcium may be supplied in any convenient form known in the art for such assays, e.g. as a salt, for example CaCl.sub.2. (Col. 2:47-49).

Thus, the Examiner's assertion that this reference teaches the present invention with the exception of the use of a polycarboxyic chelating agent to inhibit calcium interference with enzyme activity is a mischaracterization of the reference. This reference actually teaches

addition of calcium to the assay buffer at a predetermined concentration in order to minimize the deleterious effects of the calcium. It does not teach or suggest that the calcium present in the urine should be inactivated. Nor does it teach or suggest that addition of a polycarboxylic acid chelator to the urine sample inactivates calcium ions so that they cannot interfere with trypsin. It merely teaches how to reduce or eliminate the effect of the calcium in the urine by bringing the calcium concentration to a predetermined level which can then be factored out of the equation. As such, this reference teaches away from the present invention in which a chelating agent is used to complex all of the calcium present in the urine and thereby inactivate the ions.

The secondary reference teaches that the concentration of ions, e.g., calcium, in a body sample can be determined by addition of a chelator to a buffer solution which is added to the sample. Similar to the teachings of Uenoyama et al., Berry et al., discloses that if the concentration of the interfering ions is known to occur at a relatively constant concentration in the sample being tested t is possible to factor out the inhibitory effect of the ions on a particular enzyme activity.

Berry et al. also discloses that a chelator can form a complex with the free ions in a solution and reduce interference of the ions with an analytical enzyme in the sample. However, this reference does not teach or suggest that polycarboxylic chelators, such as EDTA and EGTA are capable of binding strongly enough to calcium ions to completely prevent the ions from binding to trypsin. In fact, Berry et al. limited the discussion of enzymes to transferases and hydrolases, and did not disclose the effect of chelators on any proteases, in particular, trypsin. Moreover, Berry et al. teaches use of two selective binding agents to bind the interfering ions. Berry et al. teach that a competitive exchange of the ions between a first and second selective binding agents must occur. In addition, Berry et al. teach that **the bound ions are removed from the sample**, rather than simply inactivated as in the present invention.

It is respectfully submitted that the practitioner of ordinary skill in the art would not be motivated to combine these references in the manner asserted by the Examiner. The primary reference teaches as a whole that interference of calcium ions in a urine sample is addressed by bringing the calcium concentration to a constant, predetermined level in the test sample. The secondary reference also teaches that if the concentration of the interfering ion is known, "allowance can be made for this by including an appropriate concentration of the interfering ion in standard (calibrating) solution." (Col. 3:62-66). Thus, the combination of these references teaches that interference of calcium ions on enzyme activity is effectively dealt with by adding ion to the standard (control) and sample so that both have the same amount and thus, the effect on enzyme activity is masked.

The Examiner has read out of the primary reference an essential step- **the addition of calcium to the sample is required** to bring the calcium concentration to a known, constant level- and replaced it with the teachings of the present invention. However, at best the combined cited prior art merely teaches addition of calcium to the samples to mask the inhibitory effect of the ions.

Moreover, neither the primary nor secondary reference, alone or in combination, discloses or suggests that addition of EGTA to a urine sample completely complexes the calcium ions present therein and eliminates variations between samples, without removal of the chelator-ion complexes from the sample. As such, the combined prior art does not render the claimed invention obvious.

Accordingly, the rejection of claims 1-4, 7, 9, 15 and 16 under 35 U.S.C. § 103(a) over Uenoyama et al. in view of Berry et al. is respectfully traversed.

V. Rejection of Claims 5 and 6 Under 35 U.S.C. § 103(a)

Claims 5 and 6 stand rejected under 35 U.S.C. § 103(a) as being unpatentably obvious over Uenoyama et al., in view of Berry et al., and further in view of May et al. The Examiner has applied Uenoyama and Berry as above and relies on May as teaching a diagnostic test device containing dry test reagents. The Examiner concludes that the present invention would have been obvious to one of ordinary skill in the art to use the device of May to practice the method of Uenoyama as modified by Berry.

Applicants respectfully disagree with the Examiner's conclusion.

As discussed above, the combination of Uenoyama and Berry does not teach or suggest the present invention. In particular, this combination of prior art does not teach use of a polycarboxylic chelating agent to inactivate calcium ions present in a urine sample to prevent calcium interference with trypsin. The third reference does not cure this deficiency.

May merely teaches a device containing dry test reagents. This prior art reference does not teach or suggest use of polycarboxylic chelator in a urine test sample to reduce variations between samples. Nor does this reference teach that polycarboxylic chelators completely block calcium ions from binding to trypsin. Thus, this cited reference does not cure the deficiencies of the primary and secondary references and the cited combination of prior art does not render the present invention obvious.

Accordingly, the rejection of claims 5 and 6 under 35 U.S.C. § 103(a) over the cited prior art is respectfully traversed.

VI. Rejection of Claim 10 Under 35 U.S.C. § 103(a)

Claim 10 stands rejected under 35 U.S.C. § 103(a) as allegedly being obvious over Uenoyama et al. and Berry et al as applied above, in combination with Nanbu et al. The Examiner states that Nanbu teaches use of L-amino acids as a trypsin substrate in an assay for trypsin inhibitor. The Examiner concludes, therefore, that it would have been obvious to one of ordinary skill in the art to have used the substrate taught by Nanbu in an assay taught by Uenoyama and modified by Berry.

Applicant respectfully disagrees with the Examiner's conclusion.

As discussed above, the combination of the primary and secondary references does not teach the claimed invention as set forth in claim 1. Thus, the combination of these two prior art references with Nanbu et al. does not render the invention of claim 10 obvious. The combination of art does not teach or suggest the claimed method of assaying for trypsin inhibitors in a urine sample wherein calcium in the urine sample is completely complexed with a polycarboxylic chelator to thereby eliminate calcium interference with trypsin.

Accordingly, the rejection of claim 10 under 35 U.S.C. § 103(a) over the combined cited prior art is respectfully traversed.

It is respectfully submitted that the present application, as amended above, is in condition for allowance, an early notification thereof being earnestly solicited.

To the extent necessary, a petition for an extension of time under 37 C.F.R. 1.136 is hereby made. Please charge any shortage in fees due in connection with the filing of this paper, including

extension of time fees, to Deposit Account 500417 and please credit any excess fees to such deposit account.

Respectfully submitted,

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THE IMPORTANCE OF BUFFER, SUBSTRATE, pH AND TEMPERATURE IN THE SELECTION OF A METHOD FOR TOTAL ALKALINE PHOSPHATASE ACTIVITY MEASUREMENTS WITH LEAST ISOENZYME BIAS

by

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2. Classification of Buffers.

Buffers used for measurements of ALP activity may be classified into three groups (5): 1) neutral buffers containing carbonate or barbital which are inert to the enzyme, substrate and activators, and simply buffer the reaction mixture, 2) inhibitory buffers such as glycine, which probably chelate with and perhaps remove zinc activator from the enzyme, and 3) activating buffers, such as TRIS, 2AMP, DEA, and EAE, which accept the phosphoryl product and thus serve as co-substrates in the enzyme reaction.

As described in the section on "Reaction Mechanism", trans-phosphorylation is the transfer of the phosphate groups from the substrate to certain organic chemical compounds containing an OH-group that are present in the reaction system as buffer or otherwise (10). The formation of an acceptor-phosphate as reaction product requires a two-substrate reaction, with the organic phosphate (=donor) and the alcohol (=acceptor) serving as substrates and co-substrates. The increasing ALP activity observed by increasing the alcohol concentration parallels the increased phosphate transfer activity, and suggest that the relation between enzyme reaction rate and acceptor concentration follows the Michaelis-Menten equation.

Since these aminated alcohols are phosphoryl acceptors, which also function to buffer the enzyme reaction mixture, they may be described as "phosphorylatable buffers". It is also apparent that some phosphate transfer can occur with acceptors that are not buffers, as has been noted in the presence of high concentrations of glucose and mannitol.

Charact rization of Immobilized *Escherichia coli* Alkaline Phosphatase R actors in Flow Inj ction Analysis

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The characterization of immobilized *Escherichia coli* alkaline phosphatase reactors used in flow injection analysis is reported for factors such as optimum pH, activity, ionic strength, product inhibition, and substrate specificity. The kinetics of the immobilized enzyme was studied, and mathematical descriptions were developed for the use of an immobilized enzyme packed-bed reactor to evaluate the kinetic parameters and the number of active sites on the immobilized enzyme. Suppression of phosphatase activity by orthophosphate was found to be significantly reduced, and the Michaelis–Menten constant increased when the enzyme was immobilized and packed in a reactor. Immobilized *E. coli* alkaline phosphatase exhibited similar activity at pH 8 in Tris–HCl, $NaHCO_3$, and borate–HCl buffers but slightly lower activity in NH_2H_2O–NH_4Ac buffer. The performance of the immobilized enzyme reactor was not affected by the presence of up to 10 M Mg(II), Ni(II), Cd(II), Co(II), Mn(II), Cu(II), or urea, 1 M Fe(II), or 0.1 M Fe(III) in the substrate stream. The chelating agent EDTA, however, gradually deactivated the immobilized enzyme. The periodic restoration of enzyme activity was achieved following the removal and addition of zinc ions. The immobilized *E. coli* alkaline phosphatase packed-bed reactor was used to measure the alkaline phosphatase available phosphorus content of a number of model organophosphorus compounds. *p*-Nitrophenyl phosphate showed a linear response in the range of 1.6×10^{-7}–1.6×10^{-4} M. This study forms part of a larger program to develop enzymatic systems for water quality measurement.

INTRODUCTION

The use of immobilized enzymes for chemical analysis has generated considerable interest in recent years.[1,2] One area of research activity is the use of on-line immobilized enzyme packed-bed reactors (IEPBR) in flow injection analysis (FIA).[3,4] The FIA methodology enables the conditions in an enzyme reactor to be precisely defined and provides a convenient means for characterizing immobilized enzymes for factors such as pH, temperature, activity, stability, substrate specificity, inhibition, and selection of support. Other advantages of FIA include high sample throughput and small volume requirements.

Theoretical aspects of packed-bed immobilized biocatalyst reactors have been reported by several authors.[5,6] Here a relevant model is presented for an immobilized *Escherichia coli* alkaline phosphatase reactor, and simple mathematical expressions are developed for evaluating the kinetic parameters which describe the immobilized enzyme. This kinetic approach enables one to optimize reaction conditions for an IEPBR, to predict the necessary reactor size, and to determine the linear measurement range.

Nonspecific alkaline phosphatases are the most widely recognized enzymes in aquatic systems.[7-10] They have been shown to be important in the utilization of a fraction of dissolved organic phosphorus by organisms such as microalgae and bacteria.[11] Quantification of this enzymatically hydrolyzable fraction of phosphorus is thought to be important in understanding the cycling of phosphorus in natural waters and in improving water quality management strategies. The physical, chemical, and enzymatic properties of free alkaline phosphatase from *E. coli* have been studied in detail.[12-15] The mechanisms by which this enzyme hydrolyzes substrates have been proposed by numerous authors.[16-18] In this paper, we report a study to characterize immobilized *E. coli* alkaline phosphatase for use in measuring enzymatically available phosphorus in natural and waste waters. Factors such as pH, buffer medium, substrate specificity, product inhibition, interferences, and activity restoration were studied. This study is part of a larger program to develop enzymatic systems that can be used to provide more specific measurements of water quality.

EXPERIMENTAL SECTION

Materials. *E. coli* alkaline phosphatase (EC 3.1.3.1) was purchased from United States Biochemical Corporation (Product No. 10940). CNBr-activated Sepharose 4B beads, used as the support for enzyme immobilization, were obtained from Phar-

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(2) *Biosensors—A Practical Approach*; Cass, A. E. G., Ed.; IRI Press: Oxford, 1990.
(3) Hansen, E. H. *Anal. Chim. Acta* **1988**, *216*, 257–273.
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(15) Applebury, M. L.; Johnson, B. P.; Coleman, J. E. *J. Biol. Chem.* **1970**, *245*, 4969–4976.
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(17) Reid, T. W.; Wilson, I. B. In *The Enzymes. Volume IV: Hydrolysis, Other C-N Bonds Phosphate Esters*; Boyer, P. D., Ed.; Academic: New York, 1971; Chapter 17.
(18) Xu, X.; Kantrowitz, E. R. *Biochemistry* **1991**, *30*, 7789–7795.

macia. *p*-Nitrophenyl phosphate (*p*-NPP) (disodium salt, hexahydrate, Sigma), D-glucose 6-phosphate (disodium salt, hydrate, 98%, Sigma), adenosine 5'-tripolyphosphate (disodium salt, from equine muscle, trihydrate, 99%, Sigma), sodium tripolyphosphate (Ajax), DL-α-glycerol phosphate (disodium salt, hexahydrate, 98%), sodium pyrophosphate (decahydrate, ACS reagent, Sigma), (2-aminoethyl)phosphonic acid (97%, anhydrous, Sigma), phosphonoformic acid (trisodium salt, hexahydrate, Sigma), phytic acid (magnesium potassium salt, 95%, Sigma), and bis-(*p*-nitrophenyl) phosphate (sodium salt, Sigma) were used as received in enzyme specificity assays. *p*-Nitrophenol (*p*-NP), used in the calibration of enzymatic reactions, was also from Sigma. All other chemicals were of analytical grade and were from BDH or Ajax. The pH of buffer solutions was measured with a Model PHM82 standard pH meter (Radiometer, Copenhagen). Solutions used in enzyme immobilization procedures were prepared at least 3 h before their use and stored at 4 °C to minimize enzyme deactivation. All solutions were prepared with Milli-Q reagent water (Millipore Corp.).

In the spectrophotometric FIA measurement of orthophosphate, the acidic ammonium molybdate solution and acidic tin(II) chloride solution were prepared by the method of Karlberg and Pacey.[19] The former contained 8 mM ammonium molybdate and 0.63 M concentrated sulfuric acid. The later contained 0.89 mM tin(II) chloride, 15 mM hydrazinium sulfate, and 0.5 M concentrated sulfuric acid. The 4-(2-pyridylazo)resorcinol (PAR) (disodium salt, Eastman Kodak Co.) reagent used in the Zn(II) ions measurement was 3×10^{-4} M and was prepared before use by completely dissolving the PAR in 200 mL of 7.4 M ammonia solution and then slowly adding 300 mL of 1.7 M acetic acid. All solutions used in flow injection systems were degassed whenever it was necessary.

Preparation of the Immobilized Enzyme. Coupling procedures recommended by Pharmacia[20] were employed for the immobilization of *E. coli* alkaline phosphatase. In two preparations, 0.25 g (batch 1) or 0.5 g (batch 2) of CNBr-activated Sepharose 4B, after being washed with 10 mM HCl, was mixed with 3.2 mg (batch 1) or 5 mg (batch 2) of enzymes in 2.5 mL of coupling buffer (0.1 M $NaHCO_3$, containing 0.5 M NaCl). The mixture was gently rotated end-over-end at 4 °C overnight. The gel was then washed with coupling buffer, and remaining active groups were blocked with Tris-HCl buffer (0.1 M, pH 8) for 1–2 h at room temperature. The gel was further washed with three cycles of buffers of alternating pH to remove protein. The resultant alkaline phosphatase–Sepharose gel was stored at 4 °C in Tris buffer (0.1 M, pH 8). The immobilized enzyme showed excellent stability upon storage. No change in activity was noticed after a period of 3 months.

Determination of Enzyme Activity. The activity of free alkaline phosphatase was determined by measuring the rate of *p*-NP formation spectrophotometrically. After a small aliquot of enzyme solution was added into 3 mL of Tris buffer (1.0 M, pH 8 containing 0.001 M *p*-NPP), the mixture was mixed, and the change in absorbance at 410 nm was monitored over the first 2 min. The assay was conducted at room temperature (22–25 °C). Tris buffers containing *p*-NPP ranging from 2.60×10^{-6} to 1.04×10^{-4} M were used in the determination of a Michaelis-Menten constant for the free enzyme. Tris buffers containing a given concentration of *p*-NPP but increasing amounts of orthophosphate ranging from 8.41×10^{-6} to 9.68×10^{-4} M were used for the determination of a dissociation constant of the phosphoryl enzyme in solution.

The activity of immobilized enzyme was determined in a manner similar to that for the free enzyme, except that the mixture was stirred for 3 min after the addition of a small volume of immobilized enzyme and the absorbance was measured at the end of the mixing period. The activities of batch 1 and batch 2 preparations were determined to be 8.5 and 6.7 units mL^{-1}, respectively. Coupling efficiency was about 30%. In pH-dependent assays, Tris buffers of the same concentration, but containing various concentrations of HCl, were used.

(19) Karlberg, B.; Pacey, G. E. *Flow Injection Analysis, A Practical Guide*; Elsevier: Amsterdam and New York, 1989.
(20) Product information note, Pharmacia LKB Biotechnology, 1991.

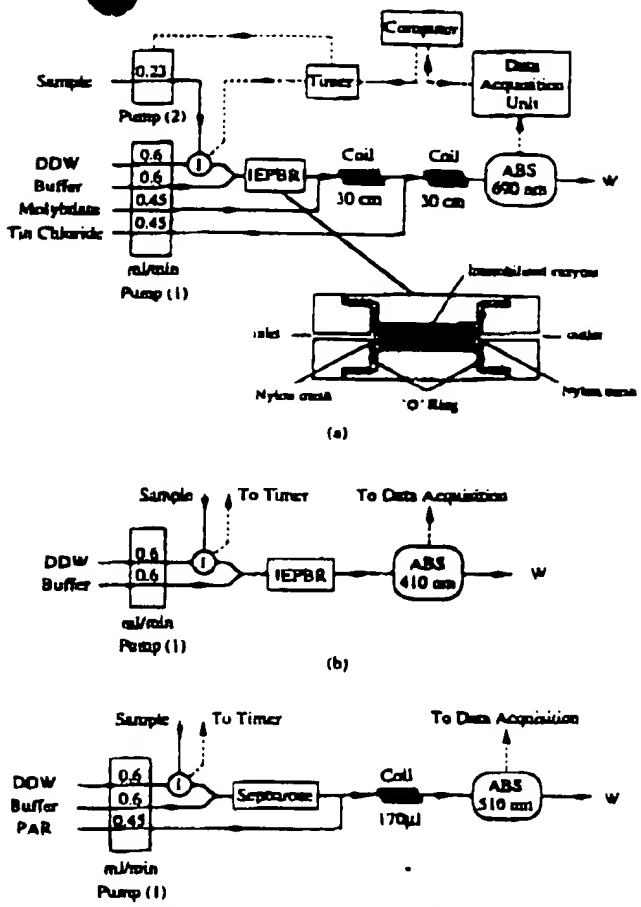

Figure 1. Manifolds for (a) the determination of APAP, (b) the characterization of the immobilized enzyme, and (c) the determination of zinc ions.

Packing the Enzyme Reactor. The enzyme bed reactors (1.1-mm i.d. × 2 mm, 1.7-mm i.d. × 5 mm, 3-mm i.d. × 20 mm, or 3-mm i.d. × 30 mm) were made from perspex (Figure 1). Both inlet and outlet were fitted with nylon mesh (20-µm pore size and 4.8-mm i.d.) to retain the immobilized enzyme beads. Polypropylene end fittings (Cheminert, VICI Valco Instruments Co., Inc.) were used to connect the reactor to the manifold. The reactors were packed by pipetting the immobilized enzyme suspension in buffer into the bed until a full bed volume was reached and allowed to settle under gravity. When not in use, the reactor columns were stored at 4 °C in 0.1 M Tris buffer at pH 8.

Flow Injection System. The system used comprised an IEPBR for enzymatic hydrolysis, a Spectroflow 757 spectrophotometer (ABI Analytical Kratos Division) with a 12-µL flow-through cell for product measurement, a Digital Peripheral 386 PC computer with a Chrom-A-Set 500 (Barspec) data acquisition unit, and a timer for process automation. A schematic diagram of the flow injection system for the study of alkaline phosphatase available phosphorus (APAP) compounds is shown in Figure 1a. An Ismatec MS-CA2 840 fixed-speed pump was used for sample delivery, and a four-channel Ismatec MS-4-Reglo 100 variable-speed pump was used for carrier and reagents delivery. Tygon pump tubes were used with these two pumps. A Rheodyne 5041 valve with an electrical actuator built in-house and a 250-µL loop was used for sample injection. Teflon tubing of 0.5-mm i.d. was used for the FIA manifold assembly. Tris buffer (0.1M, pH 8) containing 0.5 M Na_2SO_4 was used as the buffer carrier unless otherwise stated.

Orthophosphate produced by hydrolysis with alkaline phosphatase from an injected sample was detected as phosphomolybdenum blue and was measured at 880 nm. When orthophosphate is present in a sample, the analytical response measured corresponds to the sum of APAP and the dissolved reactive

phosphorus content. Characterization of immobilized alkaline phosphatase was carried out in a flow injection system consisting of a buffer stream and a water carrier line (Figure 1b) unless otherwise stated. In these experiments, *p*-NPP was used as the substrate, and the product of enzymatic hydrolysis, *p*-NP, was measured at 410 nm. A small reactor size was chosen for reasons discussed later in this article. The degree of conversion was calculated from the ratio of the FIA peak height of a substrate solution to that of *p*-NP or orthophosphate solution (depending on the detection method used) of the same concentration.

Measurement of Zn(II) Ions. In the study of the restoration of enzyme activity, a visible absorbance detection method using PAR[22] has been used for the quantification of Zn(II) ions. The reagent delivery module is shown in Figure 1c. In plotting the calibration curve of Zn(II) ions, data points from the first injection for each given concentration were used to avoid the detection error due to the accumulation of Zn(II) ions along the line. Every injection of Zn(II) solution was followed by two injections of sulfuric acid solution (pH 1) to ensure the removal of adsorbed Zn(II).

RESULTS AND DISCUSSION

Immobilized Enzyme Kinetics. Competitive inhibition of *E. coli* alkaline phosphatase by orthophosphate is well known.[16,17] Many reaction schemes have been proposed for alkaline phosphatase-catalyzed hydrolysis.[16,17] Under the present experimental conditions, a simplified version is formulated which includes a minimal number of intermediates but retains the essential features of the reaction sequence:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E\text{-}P + ROH, \qquad E\text{-}P \overset{K_{pi}}{\rightleftharpoons} E + P \tag{1}$$

where E and S represent respectively the enzyme and the substrate, ES is the enzyme–substrate complex, and E–P is the phosphoryl enzyme. The back reaction between E–P and ROH is neglected. Step k_2 is assumed to be the rate determining step. K_{pi} is the dissociation constant of E–P. A steady-state approach yields

$$v = \frac{kE_T S}{S + k_{mi}\left(1 + \frac{P_i}{K_{pi}}\right)} \tag{2}$$

where v is the rate of the enzyme reaction, E_T the enzyme activity per unit volume of enzyme–Sepharose matrix, S the concentration of the substrate, P_i the concentration of the competitive inhibitor, k ($k = k_2$) the catalytic constant, and k_{mi} ($k_{mi} = (k_{-1} + k_2)/k_1$) the Michaelis constant for the immobilized system. For the product inhibition kinetics (eq 2), eq 3 was derived to describe the substrate conversion in an ideal plug-flow immobilized enzyme reactor:[23]

$$\left(1 - \frac{k_{mi}}{K_{pi}}\right) S_0 X - k_{mi}\left(1 + \frac{S_0}{K_{pi}}\right) \ln(1 - X) = \frac{kE_T}{Q} \tag{3}$$

In this expression, S_0 is the initial concentration (M) of the substrate, X the degree of conversion, E_T the total enzyme activity (mol) in the reactor, and Q the flow rate (L min^{-1}). kE_T represents the maximum rate (mol min^{-1}) of product formation passing through the bed reactor. Since $0 \le X \le 1$, eq 3 can be expressed as

$$\left(1 - \frac{k_{mi}}{K_{pi}}\right) S_0 X - k_{mi}\left(1 + \frac{S_0}{K_{pi}}\right)\left(-X - \frac{(-X)^2}{2} + \frac{(-X)^3}{3} + \cdots + \sum_{n=3}^{\infty} \frac{(-1)^n (-X)^{n+1}}{n+1}\right) = \frac{kE_T}{Q} \tag{4}$$

When X is very small, the expansion of $\ln(1 - X)$ will be dominated by the $-X$ term. Replacing X by c_p/S_0, where c_p is the concentration (M) of the product, the approximation of eq 4 yields

$$c_p Q = \frac{kE_T S_0}{S_0 + k_{mi}} \tag{5}$$

This expression has the Michaelis–Menten kinetics form. Rearrangement of eq 5 gives

$$\frac{1}{c_p Q} = \frac{k_{mi}}{kE_T}\frac{1}{S_0} + \frac{1}{kE_T} \tag{6}$$

$$c_p Q = -k_{mi}\frac{c_p Q}{S_0} + kE_T \tag{7}$$

or

$$\frac{S_0}{c_p Q} = \frac{S_0}{kE_T} + \frac{k_{mi}}{kE_T} \tag{8}$$

These three equations (6–8) are similar to the Lineweaver–Burk reciprocal, Hanes–Woolf, and Woolf–Augustinsson–Hofstee plots for free enzymes[23] and can be used for plotting enzyme kinetic data for flow systems. It is worth noting that a number of workers[24,25] have reported that k_{mi} is a function of flow rate. The k_{mi} term includes the influence of diffusion limitation of substrates on the kinetics of immobilized enzymes. A graph of product formation as a function of substrate concentration is shown in Figure 2a. These data were used to construct the kinetic plots of $1/c_pQ$ vs $1/kE_T$, c_pQ vs c_pQ/S_0, and S_0/c_pQ vs S_0 (Figures 2b–d). The slopes and intercepts of these plots enable k_{mi} and kE_T to be determined. For an immobilized alkaline phosphatase reactor of 1.9-μL volume (1.1-mm i.d. × 2 mm) containing 8.6 units mL^{-1} of enzyme activity, we obtained values of 1.13×10^{-4} M for k_{mi} and 1.95×10^{-6} mol min^{-1} for kE_T. These values are the average of those obtained from the three plots.

When an initial concentration of orthophosphate, P_{in}, is present in the substrate solution, eq 3 no longer applies. We introduce the mass balance to a fluid element under the idealized plug-flow conditions, in which the IEPBR is characterized by a variation of component concentrations from the entrance to the exit:

$$S_0 Q\, dX = v\, dV_{im} \tag{9}$$

where dV_{im} is the increment of the volume of the enzyme–Sepharose matrix, $X = c_p/S_0$, as described earlier in the text, and v is described by eq 2. $P_i \simeq P_{in} + S_0X$ when the concentration of P·E $\ll P_{in} + S_0X$. Integration of eq 9 under the boundary conditions that $V_{im} = 0$ when $X = 0$ and $V_{im} = V_{im}$ when $X = X$ results in eq 10 for the performance of

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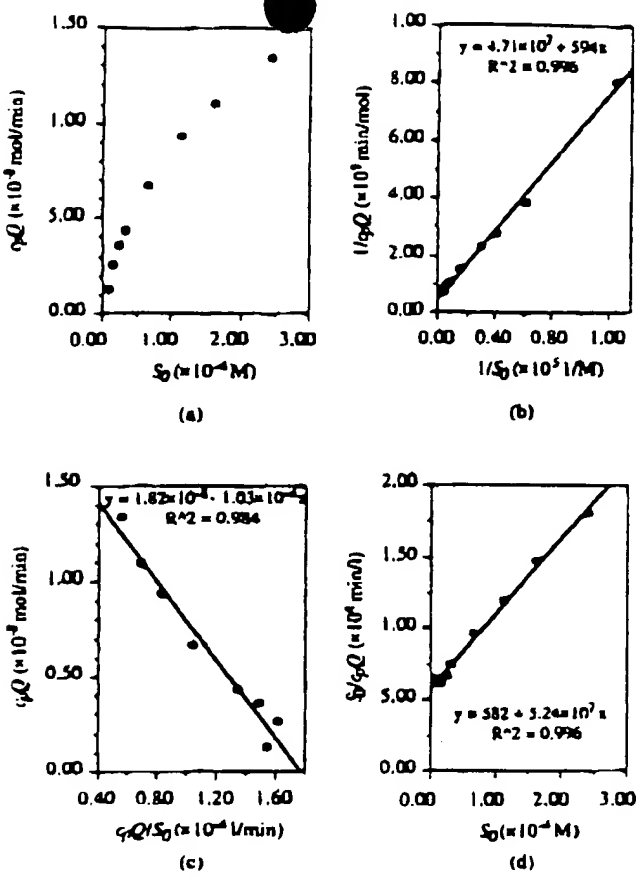

Figure 2. Kinetic plot for immobilized alkaline phosphatase based on data obtained from the FIA–IEPBR system (Figure 1b). (a) Michaelis–Menten form plot. (b) $1/c_pQ$ versus $1/S_0$. (c) c_pQ versus c_pQ/S_0, and (d) S_0/c_pQ versus S_0. *p*-NPP was used as the substrate and was ranging from 8.07×10^{-6} to 2.42×10^{-4} M. Reactor was of 1.1-mm-i.d. × 2-mm size and contained immobilized enzyme of 8.5 units mL^{-1} activity.

the reactor:

$$\left(1 - \frac{k_{mi}}{K_{pi}}\right) S_0 X - k_{mi}\left(1 + \frac{S_0}{K_{pi}} + \frac{P_{i0}}{K_{pi}}\right) \ln(1 - X) = \frac{kE_T}{Q} \quad (10)$$

where $E_T = V_R E_e$ and V_R is the total volume of enzyme–Sepharose matrix included in the reactor.

When X is very small, eq 11 is obtained, which is similar to eq 5.

$$c_p Q = \frac{kE_T S_0}{S_0 + k_{mi}\left(1 + \frac{P_{i0}}{K_{pi}}\right)} \quad (11)$$

Rearrangement of the above equation gives

$$\frac{1}{c_p Q} = \frac{k_{mi}}{kE_T S_0} + \frac{1}{kE_T} + \frac{k_{mi} P_{i0}}{kE_T S_0 K_{pi}} \quad (12)$$

A plot of $1/c_pQ$ as a function of P_{i0} at constant S_0 can therefore be used to obtain K_{pi}. From such a plot for immobilized alkaline phosphatase (Figure 3), K_{pi} was calculated to be 1.07×10^{-4} M, with the value of k_{mi} obtained from eqs 6–8. Equation 11 suggests that for orthophosphate inhibition to be neglected, the concentration of this substance must be much less than K_{pi}, that is $P_{i0}/K_{pi} \ll 1$. In many river waters, the concentration of orthophosphate is less than 1×10^{-6} M (i.e., much less than the value of K_{pi} for IEPBR), and therefore the inhibitory effect may be neglected. However, when inhibition is evident, the size of the IEPBR can always be

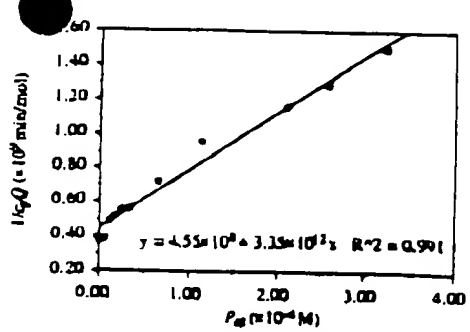

Figure 3. Kinetic plot for the orthophosphate inhibition on immobilized alkaline phosphatase based on data obtained from the FIA–IEPBR system (Figure 1b). 1.61×10^{-5} M *p*-NPP solutions containing orthophosphate ranging from 4.84×10^{-6} to 3.23×10^{-4} M were used as the substrate solutions. Reactor was of 1.1-mm-i.d. × 2-mm size and contained immobilized enzyme of 8.5 units mL^{-1} activity.

increased to achieve 100% conversion of the injected sample and offset the inhibitory effect.

Values of the Michaelis–Menten constant and the dissociation constant for free alkaline phosphatase were found to be 2.13×10^{-5} and 1.14×10^{-4} M, respectively, from the Woolf–Augustinsson–Hofstee plot of the initial reaction rate, v_i, versus v_i/S_0 and phosphate inhibition plot of $1/v_i$ versus P_{i0}. These two plots have the following regression equations: $v_i = 4.93 \times 10^{-4} - (2.14 \times 10^{-5})\, v_i/S_0$ ($R^2 = 0.981$) and $1/v_i = 2.63 \times 10^5 + (3.79 \times 10^9) P_{i0}$ ($R^2 = 0.986$). A comparison of the magnitudes of the dissociation constants for free and immobilized enzyme shows that a much lower concentration of orthophosphate causes inhibition of the free enzyme compared with that in an immobilized form and packed in a column.

Size of IEPBR. In an analytical system, it is desirable that an IEPBR operates at 100% of sample conversion, otherwise frequent calibration is necessary due to the nonlinearity of the calibration curve in the <100% conversion range and the possible loss of enzyme activity with time. An estimate of the amount of immobilized enzyme needed to provide an excess of enzyme activity on the bed can be obtained from eq 3. This shows that the total enzyme activity kE_T on an IEPBR must be greater than $Q\,(S_0 + 6.9k_{mi} + 6.9k_{mi}S_0/K_{pi})$ to obtain 99.9% of substrate conversion. The general expression for the estimation of the size of the IEBPR is

$$(kE_T)_0 \frac{V_x}{V_0} \geq Q\left(c_x + 6.9k_{mi} + \frac{6.9k_{mi}c_x}{K_{pi}}\right) \quad (13)$$

where c_x is the maximum substrate concentration (M) to be analyzed, V_x the size (μL) of the reactor required, and V_0 the size (μL) of the reactor for which the enzyme activity $(kE_T)_0$ (mol min^{-1}) is obtained. For example, a reactor of 103-μL volume is required to achieve 99.9% conversion of a sample containing 1.61×10^{-5} M *p*-NPP, assuming that k_{mi} is 1.13×10^{-4} M, K_{pi} is 1.07×10^{-4} M, and $(kE_T)_0$ is 1.95×10^{-5} mol min^{-1}, as were obtained from a reactor of 1.9-μL volume. In practice, IEPBRs were prepared with one-third more volume than theoretically necessary to ensure complete conversion and long operational lifetime.

Active Catalytic Sites on the Immobilized Enzyme. The possible number of active sites associated with free *E. coli* alkaline phosphatase has been studied by a number of researchers.[17] There has, however, been little study of the possible effect of the immobilization or the change in enzyme microenvironment on the number of binding sites. The binding of substrate to the enzyme can be considered to be a fast equilibrium, as the step k_2 shown in eq 1 is the rate-determining step. If the immobilized enzyme has n equivalent substrate binding sites with a dissociation K_s for the first molecule of S binding to any of the n vacant sites, then the

sequential interaction model can be described by the following:

$$E + S \underset{k_r}{\overset{K_1}{\rightleftharpoons}} ES \underset{2k_r}{\overset{\alpha_1 K_1}{\rightleftharpoons}} ES_2 \cdots\cdots \underset{nk_r}{\overset{\alpha_1\alpha_2\cdots\alpha_{n-1}K_1}{\rightleftharpoons}} ES_n$$

$$ES_{n-1} + P \qquad ES + P \qquad E + P \tag{14}$$

where the formation of the phosphoryl enzyme intermediate is omitted for simplicity and ES, ES_2, ..., ES_n represent enzyme–substrate complexes. Each substrate molecule that binds is assumed to make it easier for the next substrate molecule to bind, which is shown by the dissociation constant changing sequentially from K_1 to $\alpha_1\alpha_2\cdots\alpha_{n-1}K_1$ ($\alpha_1, \alpha_2, \ldots, \alpha_{n-1} < 1$) for the vacant sites. The concentrations of all enzyme–substrate complexes containing less than n molecules of substrate will be negligible at any concentration of S which is considerable to the value of K_1 if the factors $\alpha_1, \alpha_2, \ldots, \alpha_{n-1}$ are very small numbers. Under this condition, the Hill equation[23] can be employed to describe the relationship between the rate of the enzyme reaction per unit volume of enzyme–Sepharose matrix and the number of binding sites, as shown in eq 15, where $v_{max} = nk_rE_T$, $K = \alpha_1\alpha_2\cdots\alpha_{n-1}K_1$, and

$$\frac{v}{v_{max}} = \frac{S^n}{K + S^n} \tag{15}$$

k_r is the catalytic rate constant. v, E_T, and S have the same physical meanings as those described earlier.

If the formation of ES_n is not dominant, the Hill equation can still be used. However, n will no longer equal the number of active sites, but it will be the number of apparent substrate binding sites per molecule of enzyme. The smallest integer value above this apparent n value represents the minimum number of actual sites.

For IEPBR systems, integration of eq 9 with eq 15 under the boundary conditions that $V_{bed} = 0$ when $X = 0$ and $V_{bed} = V_R$ when $X = X$ yields

$$-\frac{K}{S_0^n}\frac{1}{1-n}(1-X)^{-n+1} + \frac{K}{S_0^n}\frac{1}{1-n} + X = \frac{kE_T}{S_0Q} \tag{16}$$

When X is very small, $(1-X)^{-n+1} \simeq 1 + (n-1)X$, and eq 16 becomes

$$\frac{K}{S_0^n}X + X = \frac{kE_T}{S_0Q} \tag{17}$$

With the substitution of $X = c_p/S_0$, eq 17 can be rearranged to give

$$\frac{S_0^n(kE_T - c_pQ)}{c_pQ} = K \tag{18}$$

where E_T, S_0, and c_pQ have the same physical meanings as those described in an earlier section. Equation 18 can also be written as

$$\log\frac{kE_T - c_pQ}{c_pQ} = \log K - n\log S_0 \tag{19}$$

and a plot of $\log [c_pQ/(kE_T - c_pQ)]$ versus $\log S_0$ will produce a straight line with a slope of n.

When the data from Figure 2 were replotted in this log form (Figure 4), an n value of 0.98 was obtained, which shows that the immobilized enzyme behaves as if it possessed a single substrate binding site for substrate concentrations up to 2.42×10^{-4} M. This result is similar to that reported for the free enzyme, where one active site was found at low substrate concentrations ($S \leq 10^{-4}$ M).[17]

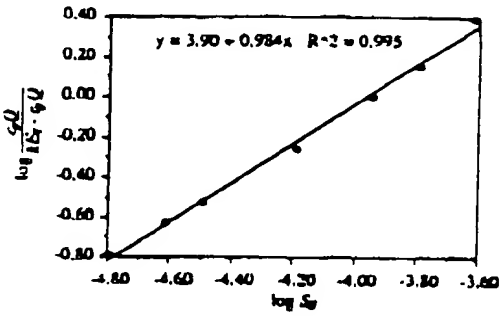

Figure 4. FIA–Hill plot for immobilized alkaline phosphatase.

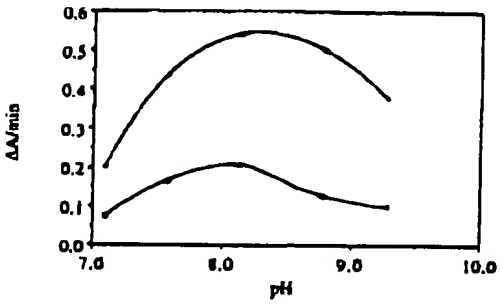

Figure 5. Effect of varying pH on the rate of conversion of *p*-NPP by (□) immobilized and (●) free *E. coli* alkaline phosphatases. One unit change in absorbance (ΔA) per minute corresponds to the generation of 61.7 μmol of *p*-NP per liter. About 0.038 mg of enzyme was involved in the assay for immobilized enzyme and 0.012 mg in the assay for free enzyme.

Effect of pH on Enzyme Activity. The optimal pH for immobilized *E. coli* alkaline phosphatase for substrate conversion was studied using the batch assay method described earlier. Maximum activity was observed to occur at approximate pH 8 (Figure 5), and for this reason buffers of pH 8 were chosen for use in the other studies reported.

Buffer Composition. The effect of buffer composition on the activity of the immobilized *E. coli* alkaline phosphatase was studied by following the degree of conversion of *p*-NPP (1.61×10^{-4} M) on a very small IEPBR. The immobilized enzyme showed the same activity in Tris-HCl (0.1 M, with 0.5 M NaCl), $NaHCO_3$ (0.1 M, with or without 0.5 M NaCl), and borate–HCl (0.0125 M) buffers at pH 8.1 ± 0.1. Its activity was about 10% higher in Tris-HCl only buffer (0.1 M, pH 8.1) and 10% lower in NH_2H_2O–NH_4Ac (0.29 M, pH 8.0) buffer. An alternative buffer, Tricine (pH 8.0) was found to inhibit the enzyme activity with time.

When Tris buffer (0.1 M, pH 8) with or without NaCl was used as the buffer stream in the flow injection system presented in Figure 1a, the calibration curves for both *p*-NPP and orthophosphate were observed to be nonlinear at low concentrations. A possible explanation for this nonlinear behavior is that orthophosphate is adsorbed onto the enzyme–Sepharose matrix as a consequence of the interaction between phosphate and enzyme as described by K_m and/or the interaction between phosphate and the Sepharose medium. The latter possibility was investigated by substituting the NaCl in the buffer stream with a salt having a higher valence anion, such as Na_2SO_4. A significant difference in behavior was observed when NaCl and Na_2SO_4 of the same ionic strength were used. Since sulfate has never been reported to be an inhibitor to alkaline phosphatase, this observation suggests that an interaction between orthophosphate and the Sepharose medium occurred. Table I shows the ratios of the signals recorded for a low orthophosphate concentration of 8.07×10^{-7} M compared to that recorded with a higher concentration of 1.61×10^{-4} M when Tris–NaCl and Tris–

Table I. Ratios of Responses of Orthophosphate of a Low Concentration (8.07×10^{-7} M) to Responses of That of a Higher Concentration (1.61×10^{-4} M) as an Indicator of Phosphate Adsorption onto the *Escherichia coli* Alkaline Phosphatase–Sepharose Matrix[a]

buffer	total ionic strength (M)	ratio calculated from peak height	ratio calculated from peak area
0.1 M Tris-HCl, pH 8 (no added salt)	0.029	<0.006	<0.01
+0.05 M NaCl	0.079	<0.006	<0.01
+0.05 M Na_2SO_4	0.18	0.006	0.02
+0.3 M Na_2SO_4	0.93	0.023	0.047
+0.5 M NaCl	0.53	0.006	0.016
+0.5 M Na_2SO_4	1.58	0.031	0.051
+1 M Na_2SO_4	3.03	0.035	0.050
+1 M NaCl	1.03	0.017	0.020
theoretical ratio[b]		0.050	0.050

[a] Sampling rate was about 20 injections per hour. Excess enzyme activity in the reactor (3-mm-i.d. × 3-cm size and with packings of 6.7 units/mL activity) ensured that it operated at 100% of substrate conversion. [b] Theoretical ratio is obtained from the fraction of 8.07×10^{-7} over 1.61×10^{-4} M.

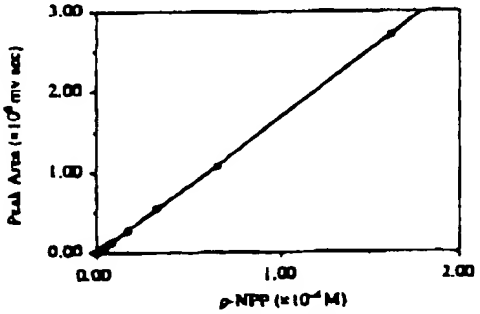

Figure 6. Typical calibration curve using FIA system 1a for *p*-NPP. Size of the reactor was 3-mm i.d. × 3 cm. Immobilized enzyme packed was of 6.7 units mL^{-1} activity [$y = 731 + (1.87 \times 10^{10})x$ ($R^2 = 1.000$)].

Na_2SO_4 buffers of various ionic strength were used. This high concentration was chosen as the reference point because the adsorption of orthophosphate on the Sepharose matrix is insignificant at the higher concentrations relative to that observed at the lower concentrations. Generally, at the higher buffer ionic strength, the experimental ratio approached the theoretical value of 0.05, indicating that negligible phosphate was adsorbed. The presence of sulfate in the buffer carrier significantly reduced the adsorption of phosphate onto the matrix and increased the ratio. On this basis, 0.1 M Tris buffer at pH 8 containing 0.5 M Na_2SO_4 was chosen as the carrier buffer in most studies.

It is also of interest to note that the ratios calculated on the basis of peak height of absorbance are consistently lower than those obtained from the peak area at the sampling rate of 20 injections per hour. The adsorption of the orthophosphate resulted in broadening of the peak and thus a lowered peak height. The peak area, however, represents the total amount of sample injected after the sample zone has passed through the reactor. For this reason, it is suggested that peak area be used as the analytical response in APAP measurement, and, thereafter, frequent calibration at low concentrations required by peak height response can be avoided. Figure 6 illustrates a typical calibration curve for *p*-NPP, where concentration ranged from 1.61×10^{-7} to 1.61×10^{-4} M. The presence of sulfate in the buffer carrier was also found to increase the sensitivity of the phosphate detection chemistry. For example, the inclusion of 0.5 M Na_2SO_4 in 0.1 M Tris

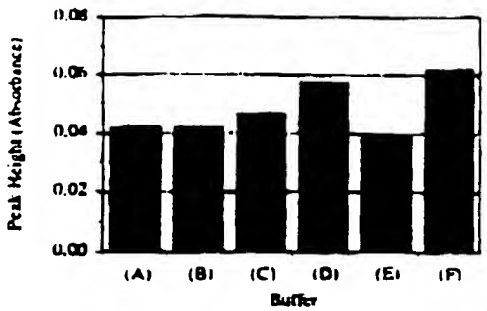

Figure 7. Effects of buffer composition on the sensitivity of APAP measurement. Concentration of *p*-NPP was 1.61×10^{-4} M. Other experimental conditions were the same as those in Figure 6. Tris buffer (0.1 M, pH 8.0) carrier containing (A) buffer alone, (B) 0.05 M NaCl, (C) 0.05 M Na_2SO_4, (D) 0.3 M Na_2SO_4, (E) 0.5 M NaCl, and (F) 0.5 M Na_2SO_4. Means ± standard deviations are shown ($N \geq 3$).

buffer increased the sensitivity by 47% (Figure 7).

The interaction between orthophosphate and the Sepharose matrix was further tested by calibrating orthophosphate using the FIA system shown in Figure 1a but with a CNBr-activated Sepharose column, which had been deactivated by washing with 10 mM HCl followed by 0.1 M Tris buffer (pH 8.0), in place of the IEPBR. The orthophosphate calibration curve followed a trend similar to that observed with an IEPBR, namely, it was curved at low concentrations when the peak height response was recorded. The effect of sulfate on the calibration curve was also similar to that noted earlier.

Substrate Specificity and Enzyme Reactivation. (1) **Adsorption of Zn(II) Ions onto Sepharose.** Alkaline phosphatase of *E. coli* is a zinc metalloenzyme.[25] It has been reported that the activity of the free enzyme can be reversibly removed and restored by the removal and addition of zinc ions at the active site.[26,27] Before investigating the possibly reversible activation and deactivation of the immobilized enzyme, it was necessary to establish whether an interaction occurs between Sepharose and Zn(II) ions which may affect the enzyme activity after the introduction of Zn(II) solution to the enzyme–Sepharose matrix. An established spectroscopic method used in high-pressure ion chromatography for determining Zn(II) was employed, and the corresponding signal as a function of Zn(II) concentration was studied with an online Sepharose column. A plateau was observed at low Zn(II) concentrations when 0.1 M Tris buffer of pH 8 or Tris buffer containing 0.5 M Na_2SO_4 was used as the buffer stream. The plot of response versus analyte extrapolated back gives abscissae intercepts from which the amount of Zn(II) in the sample adsorbed onto Sepharose matrix may be deduced. The abscissae intercepts had values of 5.7×10^{-6} and 2.1×10^{-6} M respectively in the absence and presence of sulfate. It is evident that fewer Zn(II) ions were adsorbed by the Sepharose matrix in the presence of sulfate.

(2) **Substrate Specificity.** The substrate specificity of immobilized *E. coli* alkaline phosphatase was tested with 10 different organic and condensed phosphorus compounds, selected on the basis of the different phosphorus bonds and degree of polyphosphorylation within their structures. Complete conversion of *p*-NPP, D-glucose 6-phosphate, DL-α-glycerol phosphate, sodium pyrophosphate, sodium tripolyphosphate, and adenosine 5′-triphosphate was achieved on an IEPBR containing excess enzyme activity. The immobilized alkaline phosphatase, however, displayed no activity toward substrates containing C–P bonds, such as (2-amino-

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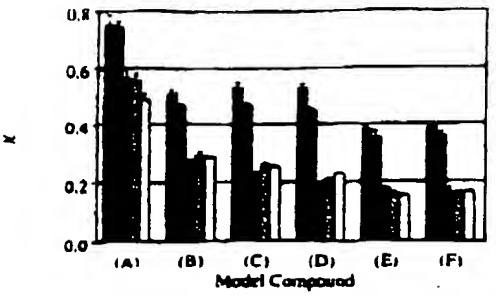

Figure 8. Enzyme activity of immobilized alkaline phosphatase for different phosphate substrates. The enzyme activity is presented by the degree of conversion on a IEPBR of limited size, 1.5-mm i.d. × 5 mm. All substrates were 1.81×10^{-4} M. (A) *p*-Nitrophenyl phosphate; (B) D-glucose 6-phosphate; (C) DL-α-glycerol phosphate; (D) sodium pyrophosphate; (E) sodium tripolyphosphate; and (F) adenosine 5′-triphosphate. The product, orthophosphate, was monitored. Means ± standard deviations are shown ($N \geq 3$); the standard deviations were calculated from the multiplicative expression for propagation of random errors.[28] (—) ■ Freshly packed with immobilized enzyme of 8.7 units mL^{-1} of activity; (—) ■ after treatment by 8 × 250 μL of 0.1 M Tris-HCl, pH 8, containing 1 mM Zn(II); (—) □ after one cycle of removal of Zn(II) by pH 1 acid solution followed by Zn(II) restoration with 0.1 M Tris-HCl pH 8, containing 1 mM $ZnSO_4$; (—) ■ after two cycles of removal and restoration of Zn(II) content; and (—) □ after six cycles of removal and restoration of Zn(II) content.

ethyl)phosphonic acid and phosphonoformic acid. Phytic acid [hexakis(dihydrogen phosphate)-*myo*-inositol] was also not hydrolyzed.

When a smaller IEPBR containing a limited amount of enzyme activity is used, the conversion degree X provides indication of the enzyme activity for a range of substrates (Figure 8). It can be seen that the activity of the immobilized enzyme for the tested compound is in the sequence of *p*-NPP > D-glucose 6-phosphate ≈ DL-α-glycerol phosphate ≈ sodium pyrophosphate > sodium triphosphate ≈ adenosine 5′-triphosphate.

E. coli alkaline phosphatase displays reversible structure and conformation changes as a function of pH.[12] Zinc ions, reported to be important for the catalytic function of alkaline phosphatase, were completely lost when the pH of the free enzyme solution was decreased to 4.0.[12] Upon increasing the pH, zinc does not bind completely until pH 6.0.[12] The influence of the dissociation and reassociation of zinc ions on the activity of immobilized alkaline phosphatase in the reactor was studied, and results are included in Figure 8. After the IEPBR experienced one cycle of acid and Zn(II) solution treatment, the enzyme activity dropped about 25% for *p*-NPP and 45–60% for the other compounds. However, no further drop in enzyme activity was observed during subsequent cycles of Zn(II) removal and restoration. A possible explanation for this change in enzyme activity is that the reactivated enzyme no longer has the same protein structure.[29] The change of enzyme activity could also be a result of interaction between Zn(II) ions of the enzyme and those adsorbed on to the Sepharose support (from the Zn(II) containing carrier), which hinder the approach of a substrate to a particular binding site. This possibility is unlikely, since there was no obvious drop in enzyme activity using a freshly packed enzyme reactor exposed to a buffer solution containing zinc ion (Figure 5). Under this latter condition, the original pattern of the quaternary structure may have remained unchanged. Possible ligand leakage from the CNBr support-ligand linkage at the low pH[30] may also result in the drop of enzyme activity.

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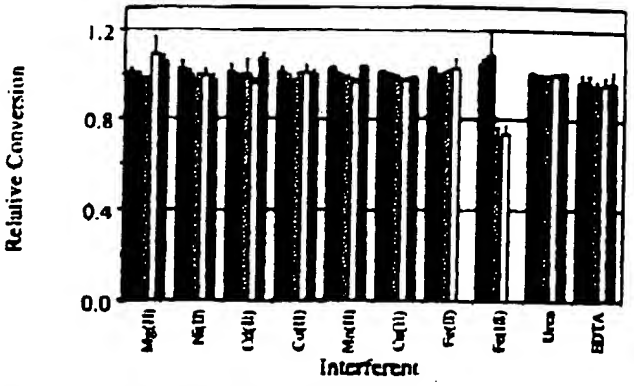

Figure 9. Effect of metal ions, EDTA, and urea on the performance of IEPBR. *p*-NPP of 1.81×10^{-4} M, containing Mg(II), Ni(II), Cd(II), Co(II), Mn(II), Cu(II), Fe(II), Fe(III), urea, or EDTA at concentration levels of (▨) 0.001 M; (▩) 0.01 M; (■) 0.1 M; (□) 1 M; and (▩) 10 M, was used as the substrate, and the product, *p*-NP was monitored. When the concentration of Fe(II) and Fe(III) reached 10 M in the substrate solution, the color of the metal ion solutions themselves interfered with *p*-NP detection severely, and therefore data at this level are not included. Size of the IEPBR was 3-mm i.d. × 2 cm, and activity of the packing was 8.7 units/mL. Data given are relative to those of *p*-NPP. Means ± standard deviations are shown ($N \geq 3$).

Although a certain reduction in enzyme activity was observed following the removal and addition of zinc ions, the acid–Zn(II) treatment procedure raises the possibility that alkaline phosphatase reactors could be periodically cleaned and enzymatic activity restored. Furthermore, the activity drop problem may well be overcome by employing excess activity in a reactor.

(3) Effect of Metal Ions, EDTA, and Urea on the Immobilized Enzyme Activity. As noted, *E. coli* alkaline phosphatase is a zinc metalloprotein. Other Me(II)–apophosphatase complexes (Me(II) = Ni(II), Cd(II), Co(II), Mn(II)) have also been reported,[31] and only Co(II) alkaline phosphatase is reported to be active. Competition between Zn(II) and other metal ions for the metal binding sites on the enzyme may result in the replacement of Zn(II) and a reduced enzyme activity. The influence of Mg(II), Ni(II), Cd(II), Co(II), Mn(II), Cu(II), Fe(II), and Fe(III) on the performance of the immobilized alkaline phosphatase reactor was therefore investigated (Figure 9). No decrease in the recovery of *p*-NPP was observed upon the inclusion of Mg(II), Ni(II), Cd(II), Co(II), Mn(II), or Cu(II) at concentrations of 0.001–10 M in the substrate solution. When Fe(II) was present in the sample, a yellow color developed on the IEPBR with time. However, the recovery of the substrate was not affected. In the presence of Fe(III), a double peak appeared over the Fe(III) concentration range of 0.1–1 M. It was recognized from the retention time that the first peak corresponded to the color of the aqueous ferric solution itself and the second peak was correlated with the product of the enzymatic hydrolysis. The recovery of *p*-NPP over this Fe(III) concentration range was significantly reduced. A possible explanation for the observed decrease in the recovery is that those Fe(III) ions adsorbed on the Sepharose medium and present in the sample stream compete with the immobilized enzyme for binding the substrate compound, which reduced the amount of the substrate thus available for the enzymatic reaction. The adsorption of Fe(III) ions onto the enzyme–Sepharose matrix was indicated by a dark yellow color developing along the length of the IEPBR with increasing number of injections. The effects noted with Fe(II) can be explained by Fe(II) being adsorbed by the Sepharose and then gradually being oxidized

(31) Lazdunski, C.; Petitclerc, C.; Lazdunski, M. *Eur. J. Biochem.* **1969**, *8*, 610–617.

to Fe(III) by oxygen pres[redacted] the mobile phase. The reduced recovery could also result from the replacement of the Zn(II) by Fe(III) on the binding sites, which subsequently deactivated the enzyme. However, this possibility was ruled out, since the recovery of *p*-NPP was quickly restored after Fe(III) was removed from the sample solution.

The effects of chelating agents, such as EDTA and urea, on the performance of the IEPBR were also tested (Figure 9). The recovery of *p*-NPP was not affected by the presence of urea up to a concentration of 10 M. However, the presence of EDTA gradually deactivated the enzyme with a decrease in conversion to less than 75% after 15 injections of 10 M EDTA.

The enzyme deactivation and reactivation method discussed previously was successfully applied to wash the metal ion contaminants off the reactor and restore enzyme activity. The activity of an EDTA-treated IEPBR was also successfully restored.

CONCLUSIONS

The present study has demonstrated the application of FIA for characterising immobilised enzymes. It has provided information on the kinetic, physical, and chemical factors affecting [redacted] use of immobilised *E. coli* alkaline phosphatase in APAP analysis. The evaluation of kinetic parameters for the immobilised enzymes was undertaken under conditions where only a small proportion of the substrate was converted. An immobilised *E. coli* alkaline phosphatase packed-bed reactor has been shown to be suitable for analytical use. Microbial growth or heavy metal contamination problems, which are most frequently encountered in field analysis, can possibly be solved by an enzyme activity restoring procedure. Enzymatic assays involving the use of IEPBRs have the potential to be a useful tool in water quality management by providing real-time data on "biologically available" forms of nutrients.

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